Detection of *Vibrio parahaemolyticus* with a Chromogenic Medium

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ABSTRACT

Vibrio parahaemolyticus is a human pathogen that occurs naturally in the marine environment and fisheries products, particularly shellfish. The commonly used medium, thiosulfate-citrate-bile salts-sucrose agar (TCBS), for detecting this pathogen can not differentiate growth of *V. parahaemolyticus* from *Vibrio vulnificus* or *Vibrio mimicus*. Therefore, presumptive positive colonies formed on TCBS need to be confirmed with biochemical tests. This study evaluated a newly developed chromogenic medium, Bio-Chrome Vibrio medium (BCVM), for specific detection of *V. parahaemolyticus* based on formation of distinct purple colonies on the medium. When pure cultures of *V. parahaemolyticus* were tested with BCVM, all 148 strains grew with 145 of them produced purple colonies on the medium. Growth of *Vibrio vulnificus* or *Vibrio mimicus* on BCVM was easily identified by formation of blue green colonies while growth of *Salmonella, Shigella, Escherichia coli, Enterobacter cloacae, Yersinia enterocolitica,* and *Aeromonas* was totally inhibited. The BCVM was compared with TCBS for detecting *V. parahaemolyticus* in 296 seawater, sediment, and oyster samples. Higher false positive and false negative results were obtained by TCBS than BCVM. The accuracies of detecting *V. parahaemolyticus* were higher for BCVM (84%) than for TCBS (54%). BCVM was more specific and accurate than TCBS for *V. parahaemolyticus* detection.

Key words: *Vibrio parahaemolyticus*, Chromogenic medium, TCBS, Pathogen detection, Food safety.

INTRODUCTION

Vibrio parahaemolyticus is a seafood-borne pathogen that causes worldwide health problems associated with seafood consumption (Liston, 1990; Rippey, 1994; Kaysner and DePaola, 2001). This organism occurs naturally in coastal waters and is frequently found in molluscan shellfish, particularly oysters (Bej *et al.*, 1999; Wong *et al.*, 1999). Consumption of raw oysters contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, vomiting and abdominal cramps. The infection may also cause life-threatening septicemia in persons having underlying medical conditions such as liver disease or immune disorders. To minimize the risks of *V. parahaemolyticus* infections associated with seafood consumption, effective post-harvest processes for eliminating *V. parahaemolyticus* in oysters and specific methods for detecting

V. parahaemolyticus need to be developed.

One of the commonly used methods for detecting *V. parahaemolyticus* in foods is the most probable number (MPN) procedure using thiosulfate-citrate-bile salts-sucrose agar (TCBS) (USFDA, 1998). However, this method is very labor-intensive and time-consuming because TCBS can not differentiate growth of *V. parahaemolyticus* from some strains of *Vibrio vulnificus* or *Vibrio mimicus*. Growth of *V. parahaemolyticus* as well as a few *V. vulnificus* and *V. mimicus* all appears as green or blue-green colonies on TCBS. Therefore, several presumptive positive colonies formed on a TCBS plate need to be confirmed by biochemical tests and the final results may not be available for 5 to 8 days.

Many methods, including molecular biology based polymerase chain reaction (PCR) and DNA hybridization (Nishibuchi *et al.*, 1985, 1986; Tada *et al.*, 1992; Yamamoto *et al.*, 1992, Bej *et al.*, 1999; McCarthy *et al.*, 1999), DNA probe combined with hydrophobic grid membrane filters (Banerjee *et al.*, 2002), and direct-plating procedures using non-radioactive DNA probes to detect the thermolabile hemolysin gene (*tl*) of *V. parahaemolyticus* (Gooch *et al.*, 2001), have been developed for rapid and specific detection of *V. parahaemolyticus*. However, these methods are much more complicate than the MPN method because of the involvement of DNA isolation, PCR primers preparation, DNA probes synthesis, and colony hybridization. In addition, special instruments and skilled technicians are essential for conducting the tests. Development of a simple and easy to use method for detecting *V. parahaemolyticus* would allow laboratory workers with no or limited microbiological training to perform the analyses.

Recently, a chromogenic medium (Bio-Chrome Vibrio medium) was developed for differentiating *V. parahaemolyticus* from other *Vibrio* species based on formation of distinct purple colonies by *V. parahaemolyticus* on the medium. Growth of other *Vibrio* species such as *V. vulnificus, V. mimicus,* and *V. cholerae* on the medium can easily be differentiated from growth of *V. parahaemolyticus* based on the formation of blue-green colonies. Although this newly developed chromogenic medium may provide an opportunity to reduce labor and time involved in *V. parahaemolyticus* detection, its application in detecting *V. parahaemolyticus* in foods and environmental samples needs to be validated. This study investigated the specificity and accuracy of this chromogenic medium for detecting *V. parahaemolyticus* in raw oysters and environmental samples.

MATERIALS AND METHODS

Bacterial cultures including 179 strains of Vibrio species (parahaemolyticus, vulnificus, cholerae, mimicus, hollisae, alginolyticus, fluvialis, and furnissii) and 42 strains of non-Vibrio bacteria, including Salmonella (choleraesuis, lansing, arizonae, havanna, enteritidis, oranienburg, stanley, houten, and meleagridis), Shigella (sonnei and flexneri), Aeromonas (hydrophila, veronii, sobria, and caviae), Enterobacter cloacae, Escherichia coli, Yersinia enterocolitica were used in this study.

Non-*Vibrio* bacteria were individually enriched twice in tryptic soy broth (TSB, Difco Laboratories, Detroit, Michigan, U.S.A.) at 37°C overnight and streaked onto both Bio-Chrome Vibrio medium (BCVM, BioMedix, Pomona, California, U.S.A.) and TCBS (Difco) plates for their abilities to grow on the media. Strains of *Vibrio* spp were individually enriched twice overnight in TSB supplemented with 1.5% NaCl at 37°C and streaked onto BCVM and TCBS plates to determine the specificity of BCVM for *V. parahaemolyticus* detection. All plates were incubated at 35-37°C for 18-24 hr and examined for growth and production of colored colonies.

A total of 296 samples (148 seawater, 74 oyster and 74 sediment) were collected from three oyster farms in States of Oregon and Washington. Freshly harvested oysters were briefly rinsed with estuarine water, shucked at the oyster plant, and placed in a plastic jar. Seawater and sediment samples were collected with sterile scoops and kept in sterile sampling containers. All samples were shipped in a cooler with ice to the OSU Seafood Lab. Presence of *V. parahaemolyticus* in samples were determined by the 3-tube MPN method according to procedures of United States Food and Drug Administration's Bacteriological Analytical Manual using both BCVM and TCBS plates. Presumptive positive colonies appeared on either TCBS or BCVM plates were confirmed as *V. parahaemolyticus* with MicroStation Microbial Identification System (BioLog, Hayward, CA, U.S.A.) according to manufacturer's instruction.

A multiplex polymerase chain reaction (PCR) targeting the *tl* (450 bp), *tdh* (269 bp) and *trh* (500 bp) genes in *V. parahaemolyticus* cells was used as a reference method for confirming *V. parahaemolyticus* in presumptive positive samples (Bej *et al.*, 1999; Kaysner and DePaola, 2001). The oligonucleotide primers for the *tl* (L-*tl*: 5'-aaa gcg gat tat gca gaa gca ctg-3' and R-*tl*: 5'-gct act ttc tag cat ttt ctc tgc-3'), the *tdh* (L-*tdh*: 5'-gta aag gtc tct gac ttt tgg ac-3' and R-*tdh*: 5'-tgg aat aga acc ttc atc ttc acc-3') and the *trh* (L-*trh*: 5'-ttg gct tcg ata ttt tca gta tct-3' and R-*trh*: 5'-cat aac aaa cat atg ccc att tcc g-3') genes were commercially synthesized (Integrated DNA Technologies, Coralville, IA, U.S.A.) according to the nucleotide sequences reported by Nishibuchi and Kaper (1985), Taniguchi *et al.* (1985) and Honda and Iida (1993),

Results obtained from the MPN method were compared with results obtained from PCR analysis. False-positive and false-negative results obtained by both BCVM and TCBS media were calculated based on PCR confirmed results. The accuracy of BCVM or TCBS for detecting *V. parahaemolyticus* was determined as 100 × [True Positive/(True Positive + False Positive + False Negative)]. A true positive result was defined as a positive result for *V. parahaemolyticus* that was confirmed by both PCR and the MicroStation System.

RESULTS

Growth of *Salmonella, Shigella, Enterobacter, Aeromonas, E. coli,* and *Yersinia* were completely inhibited by TCBS. Similar results were obtained by BCVM except for one strain of *Aeromonas hydrophila,* which grew and formed white colonies on the medium. All 148 strains of *V. parahaemolyticus* grew on BCVM and 145 of them produced purple colonies (Table 1). No purple colonies were produced on BCVM by other 31 *Vibrio* spp, except one strain of *V. fluvialis*. Growth of *V. cholerae, V. mimicus* and *V. vulnificus* on BCVM all produced blue-green colonies. Other *Vibrio* spp including *V. alginolyticus, V. furnissii,* and *V. hollisae* were either unable to grow or produced white colonies on BCVM.

Detection of *V. parahaemolyticus* in seawater, sediment and oyster with BCVM and TCBS is shown in Table 2. Among the 296

samples tested, 136 samples gave presumptive positive results on TCBS while 92 samples produced presumptive positive results on BCVM. The multiplex polymerase chain reaction (PCR) and biochemical tests (MicroStation System) confirmed that 77 samples (mainly seawater and sediment) were positive for *V. parahaemolyticus*. A total of 65 false results (62 false-positive and 3 false-negative) were observed for TCBS while only 15 (all false-positive) were reported for BCVM. The accuracies of TCBS and BCVM for detecting *V. parahaemolyticus* were determined as 54% and 84%, respectively (Table 3).

DISCUSSION

The selectivity of BCVM for detecting *Vibrio* spp was comparable to that of TCBS. Both media inhibited growth of many Gram-negative bacteria but allowed *Vibrio* bacteria to grow. However, BCVM was more specific than TCBS for *V. parahaemolyticus* detection. Growth of *V. vulnificus* and *V. mimicus* on TCBS that could not be distinguished from growth of *V. parahaemolyticus* could easily be identified on BCVM as blue-green colonies.

Table 1. Growth of *Vibrio* bacteria on Bio-Chrome Vibrio medium (BCVM) and thiosulfate-citrate-bile salts-sucrose agar (TCBS).

Vibrio spp.	No. of strains	BCVM	TCBS
V. alginolyticus	2	white	yellow
V. cholerae	13	blue green	yellow
V. fluvialis	1	purple	yellow
V. furnissii	1	white	yellow
V. hollisae	2	no growth	no growth
V. mimicus	2	blue green	green
V. parahaemolyticus	145	purple	green
	3	white	green
V. vulnificus	7	blue green	green
	2	no growth	green
	1	blue green	yellow

Table 2. Comparison of TCBS and BCVM with polymerase chain reaction (PCR) for detecting *V. parahaemolyticus*.

Test	Seawater	Sediment	Oyster	Total
TCBS (presumptive positive)	62	43	31	136
BCVM (presumptive positive)	41	35	16	92
PCR (positive)	32	33	12	77

Table 3. Accuracies of TCBS and BCVM for detecting *V. parahaemolyticus*.

Medium	Presumptive positive	False positive	False negative	Accuracy[a] (%)
TCBS	136	62	3	54
BCVM	92	15	0	84

[a] Accuracy (%) = [True Positive/(True Positive + False Positive + False Negative)] × 100. True positive = 77 (Confirmed by PCR and MicroStation System).

It is well known that TCBS may not differentiate growth of *V. parahaemolyticus* from some strains of *V. vulnificus* and *V. mimicus*. In this study, most of the bacteria producing false-positive results on TCBS were identified as *V. cholerae*, *V. mimicus*, or *V. vulnificus* (data not shown). However, none of the false-positive results obtained by BCVM were caused by any of these three species. *Vibrio vulnificus*, *V. cholerae*, and *V. mimicus* all appeared on the BCVM plate as blue-green colonies.

The formation of purple colonies by *V. parahaemolyticus* on BCVM seems to be a unique characteristic of the chromogenic medium. This study found that 98% of the 148 *V. parahaemolyticus* strains tested were able to produce purple colonies on BCVM. Although BCVM is not a perfect medium for detecting *V. parahaemolyticus*, it offers an advantage over TCBS for *V. parahaemolyticus* detection by being able to distinguish growth of *V. vulnificus* and *V. mimicus* from growth of *V. parahaemolyticus*. This is evidenced by lower false-positive and false-negative results obtained by BCVM than by TCBS. Since presumptive positive results obtained by TCBS needs to be confirmed with biochemical tests, using BCVM instead of TCBS resulted in a reduction of 44 confirmation tests (32% reduction). This result clearly indicates that BCVM can be used to reduce time and labor involved in *V. parahaemolyticus* detection.

Despite its higher specificity and accuracy than TCBS for *V. parahaemolyticus* detection, BCVM was not a perfect medium for detecting the organism. False-positive results were obtained from growth of some bacteria that were not *V. parahaemolyticus* (Table 3). Several bacteria including *Vibrio carcariae*, *Vibrio damsela*, *Vibrio fluvialis*, and *Enterococcus faecalis* were identified from samples that produced false-positive results on both TCBS and BCVM. However, there were a few bacterial isolates that appeared as presumptive positive colonies on both media but could not be identified by the MicroStation microbiological identification system. In addition, we were unable to identify the bacteria in 6 samples (3 seawater, 2 oyster and 1 sediment) that caused false-positive results only on BCVM. However, none of those unidentified bacteria were *V. parahaemolyticus* as determined by PCR analysis.

In addition to false-positive results, false-negative results were obtained for 3 samples by TCBS. All those 3 samples were found to contain very low levels of *V. parahaemolyticus* (<10 MPN/g), but were detected by BCVM and confirmed by PCR. It is believed that these false-negative results were obtained because the populations of *V. parahaemolyticus* in those samples accounted for only a very small portion of the total bacterial population. The green colonies produced by *V. parahaemolyticus* on TCBS were probably masked by a cluster of yellow colonies formed by other bacteria. However, the production of purple colonies on BCVM by *V. parahaemolyticus* in those samples was not affected by the large number of other bacteria. This result indicates that BCVM is more accurate in detecting low levels of *V. parahaemolyticus*.

It was interesting to observe that growth of *V. fluvialis* on BCVM also resulted in formation of purple colonies. It is not clear whether all strains of *V. fluvialis* will produce purple colonies on BCVM because only one strain of *V. fluvialis* was available for test in this study. Further investigation needs to be conducted to determine if all strains of *V.*

fluvialis would produce purple colonies on BCVM. Based on limited information obtained from this study, it appears that TCBS would be a better choice for *V. parahaemolyticus* detection if a large number of *V. fluvialis* is expected in samples. On the contrary, BCVM will work better than TCBS for *V. parahaemolyticus* detection when *V. vulnificus* or *V. mimicus* is expected in samples.

CONCLUSIONS

The Bio-Chrome Vibrio medium is more specific and accurate than TCBS for detecting *V. parahaemolyticus*. It can differentiate growth of *V. parahaemolyticus* from growth of *V. vulnificus* and *V. mimicus* and provides an opportunity to reduce time and labor involved in *V. parahaemolyticus* detection.

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